Side population cells in anaplastic thyroid cancer and normal thyroid.

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Running title: SP cells in normal and ATC thyroid cell lines

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ABSTRACT
Comparison of studies of cells derived from normal and pathological tissues of the same organ can be fraught with difficulties, particular with cancer where a number of different diseases are considered cancer within the same tissue. In the thyroid, there are 4 main types of cancer, three of which arise from follicular epithelial cells; papillary and follicular which are classified as differentiated, and anaplastic which is classified as undifferentiated.
One assay that can be utilised for isolation of cancer stem cells is the side population (SP) assay. However, SP studies have been limited in part due to lack of optimal isolation strategies and in the case of anaplastic thyroid cancer (ATC) are further compounded by lack of access to ATC tumors. We have used thyroid cell lines to determine the optimal conditions to isolate viable SP cells. We then compared SP cells and NSP cells (bulk tumour cells without the SP) of a normal thyroid cell line N-thy ori-3-1 and an anaplastic thyroid cancer cell line SW1736 and showed that both SP cell populations displayed higher levels of stem cell characteristics than the NSP. When we compared SP cells of the N-thy ori-3-1 and the SW1736, the SW1736 SP had a higher colony forming potential, expressed higher levels of stem cell markers and CXCR4 and where more migratory and invasive, invasiveness increasing in response to CXCL12.
This is the first report showing functional differences between ATC SP and normal thyroid SP and could lead to the identification of new therapeutic targets to treat ATC.

Introduction

While it is now established for many types of cancer that cancer stem cells (CSC) are involved in progression to a more aggressive status, less is known about the function of CSC in thyroid cancer. The side population (SP) assay in cancer studies is a technique that has been used to isolate cells rich in CSCs. It is based on the ability of SP cells to efflux vital dyes in combination with dual wavelength flow cytometry (FACS). However, poorly controlled experiments, and differences in SP assay conditions, can all impact negatively on the interpretation of SP studies. It has been reported that differences in the SP assay conditions occur even for cell lines and primary cells isolated from the same type of cancer.

In staining of SP derived from breast cancer cell lines and fine needle aspirates from palpable breast tumors, the same concentration of Hoechst 33342 dye was used for the breast cancer cell lines MDA-MB-231 and MCF7, but this had to be halved to identify SP cells in the aspirates taken from palpable breast tumors. In addition, while the SP phenotype of both cell lines was confirmed using the ABCG2 specific inhibitor, Fumitremorgin C (FTC), the more general calcium channel blocker verapamil was used to confirm the SP phenotype of the fine needle aspirate cells [1]. In ovarian cancer it has been reported that two distinct ovarian cancer cell lines were stained with identical concentrations of Hoechst 33342 dye, while to identify SP cells in ovarian ascitic fluid the concentration of dye and inhibitor used had to be reduce compared to that used for the cell lines [2]. In the thyroid SP cells have been reported to be present in the thyroid tissue of mice and were identified based on using 3 µg/ml of Hoechst dye for 90 minutes, and 150 µM verapamil to confirm the phenotype [3]. However, in a more recent study mouse thyroid SP cell were identified using 5µg/ml Hoechst 33352 dye for 60 minutes, while the SP cell phenotype was still confirmed using 150µm verapamil [4]. While for SP cells identified in thyrocyte cultures, established from nodular goiters of humans, 5µg/ml of Hoechst dye was used for 90 minutes and 50µM of verapamil was used to confirm the SP cell phenotype [5]. In a study of human thyroid cancer cell lines (anaplastic, papillary and follicular) the protocol used 5µg/ml Hoechst for 90 minutes incubation, and 50µg/ml verapamil to confirm the SP phenotype [6]. While in a more recent study on 3 human thyroid anaplastic cancer (ATC) cell lines, all 3 were stained with 5 µg/ml Hoechst dye for 120 minutes [7]. It should be noted that of the cell lines used in [6], one of the papillary and the follicular cell line have been called into question as to their suitability as models of thyroid cancers [8]. However, the variable conditions used amongst mouse thyroid SP cell studies, and amongst studies of human thyroid cell lines makes comparison between different studies difficult and often no rationale is given for the conditions employed. Like many cancers, thyroid cancer consists of several types that are quite distinct and we have hypothesized that it is unlikely that a 'one size fits all' approach can be used to best isolate SP cells from these distinct thyroid cancers.

In this study we wanted to optimize the SP assay within a single study for isolation of SP cells from both normal and thyroid cancer cell lines derived from follicular epithelial cells, including cells lines that are models for papillary and ATC, with a particularly focus on ATC, as it is the least common form of thyroid cancer and has an extremely poor prognostic outcome. Studies of ATC stem cells will prove extremely valuable in understanding how these cells differ from normal thyroid stem cells, an issue that needs clarification if we are to safely target this aggressive cancer. We have examined the concentration of Hoechst 33342 dye, incubation contact time of cells with Hoechst dye, ABC transporter expression and impact of two transporter inhibitors fumitremorgin C and verapamil at different concentrations on cells of the normal human N-thy ori-3-1 thyroid follicular epithelial cell line, BCPAP a papillary thyroid carcinoma and SW1736 an anaplastic thyroid cancer cell line. In addition we have looked at the impact of cell culture confluency. Then using these optimised conditions we have sorted viable SP and NSP cells from the human N-thy ori-3-1 and SW1736 cells lines and examined the 'stemness' of the SP cells compared to the NSP cells. We then further compared the invasive and migratory potential of the normal and ATC SP and the influence of the CXCR4/CXCL12 axis.

Results.

Optimal Hoechst 33342 dye and incubation time for SP assay

The optimal conditions for the SP assay for normal human follicular thyroid cell line N-thy ori-3-1, papillary thyroid cancer-derived cell line BCPAP and anaplastic thyroid cancer cell line SW1736 were determined using sequential steps summarised in Figure 1.

To determine optimal concentrations of Hoechst 33342 dye (data not shown) and the length of incubation period, the thyroid cell lines were incubated with different concentrations of Hoechst dye for 45, 60 and 90 minutes at 37^0C (representative FACS plots for all cell lines at all incubation times are shown in Supplementary Figure 1). The optimal set of conditions for each cell line are shown in (Figure 2A), while in (Figure 2 B) representative SP FACS plots are shown for all 3 cells lines using these optimal conditions. The G0/G1 subpopulation is contained within a well-defined area of intense fluorescence due to intracellular dye accumulation and the SP population appears as a 'tail' in the left lower quadrant of the plot. The SP phenotype was confirmed in each case by addition of the optimum concentration of verapamil (100μM), note the absence of SP cells in the gated region of the inhibited samples (Figure 2B). The highest SP percentage was observed in N-thy ori-3-1 when the cells were incubated for 90 minutes with 7μg/ml of Hoechst 33342 dye (based on n=3 biological replicates the mean live cell population=71.1%, mean SP=1.12%, data not shown) while for the SW1736 cell line, Hoechst concentration was reduced to 5μg/ml to improve SP yield (based on n=3 biological replicates the mean SP= 0.85% with 7μg/ml of Hoechst dye versus mean SP= 1.15% with 5μg/ml of Hoechst dye, mean live cell population= 68% data not shown) and for the BCPAP cell line, both Hoechst concentration and incubation times were lowered to reduce cell death rate based on n=3 biological replicates the mean SP=0.53% and mean live cell population=63.7% when incubated with 5μg/ml of Hoechst for 60 minutes versus mean SP=0.1% and mean live cell population=43.8% when the cells were incubated with 7μg/ml of Hoechst for 90 minutes (data not shown).

Thyroid cell lines express mRNA for ABC transporters and stem cell markers

To select the appropriate ABC-transporter inhibitor for use in confirmation of SP cell phenotype, semi-quantitative (sq-PCR) was performed on each cell line type. All 3 cell lines expressed ABCG2 mRNA and N-thy ori-3-1 and BCPAP cell lines expressed ABCB1 mRNA, while SW1736 did not express this transporter (Figure 3A). GapdH was used as a loading control. We also examined all 3 bulk cell lines using quantitative PCR (qPCR) for expression of the stem cell markers NANOG, OCT4 and SOX2. We observed that all express mRNA for all 3 genes but that gene expression was only significantly different between the N-thy ori-3-1 cells and the SW1736 cells with SOX2 and OCT4 expression being significantly higher in the SW1736 (Supplementary Figure 2).

ABC-transporter Inhibitor Optimisation for confirmation of SP Phenotype

A comparison of inhibition of vital dye efflux potential is shown (Figure 3). The ABCG2 –specific transporter inhibitor FTC and verapamil, which targets ABCB1 transporters but also has affinity for other ABC-transporters including ABCG2, was undertaken for all cell lines. The effect of increasing concentration of verapamil on cell viability of bulk cells (Figure 3B), revealed a sharp rise in the cell death rate at 150uM and above for the N-thy ori-3-1 and BCPAP cell lines, therefore 50μM and 100μM were used in the optimisation protocol as shown for the N-thy ori-3-1 cell line (Figure 3C). In N-thy ori-3-1 full inhibition was achieved with verapamil when the dose was titrated up to 100μM (SP=0%), while a residual SP population could still be observed in the presence of 50μM (SP=0.1%). Whilst using FTC as an inhibitor, a small population 0.1% still remained after FTC was used at a concentration of 70μM in the N-thy ori-3-1 cell line (Figure 3D). At these concentrations the cell viability was 81% for verapamil versus 72% for FTC. Verapamil and FTC used in the SP assay for the BCPAP and SW1736 cell line produced similar results to the N-thy ori-3-1.

In SW1736, cell death in the bulk cells increased but at a much lower rate at 100μM to 200μM when using verapamil compared with the other cell lines (Figure 3C). However, 100μM concentration still prevented Hoechst efflux by the SP cells (Figure 3D).

Cell confluency impacts on SP cell yield

In all cell lines, cell populations with lower confluency resulted in the highest SP percentage. The SP percentages drop from (0.3-0.1%) in N-Thy ori-3-1, (2.6-0.1%) in BCPAP and (4.3-0.9%) in the SW1736 (Figure 4).

Thyroid SP cells of the N-thy ori-3-1 and SW1736 express stem cell markers.

Immunocytochemical (ICC) analysis results determined based on the mean fluorescence intensity (MFI) of cells expressing NANOG, OCT4 and SOX2 revealed that for both cells lines some SP and NSP cells expressed NANOG, OCT4 and SOX2 (Figure 5). However, for both cell lines NANOG expression was significantly higher in the SP vs. the NSP ($p<0.01$) but there was no significant difference in NANOG expression between the SP of both cell lines (Figure 5A). While OCT4 expression in both cell line SP was significantly higher in the SP vs. the NSP (for N-thy ori-3-1 $p<0.05$ and for SW1736 $p<0.001$). In addition the expression of OCT4 was significantly higher in the SW1736 SP versus. the N-thy ori-3-1 SP ($p<0.001$) (Figure 5B). SOX2 expression was significantly higher in the SP vs the NSP for both cells lines (N-thy ori-3-1 $p<0.001$ and SW1736 $p<0.001$). However there was no significant difference in SOX2 expression between the two SP cell populations (Figure 5C). Representative immunofluorescence images of ICC staining for NANOG (Figure 5 D), OCT4 (Figure 5E) and SOX2 (Figure 5F) for both cell lines SP and NSP show sorted cells stained for expression of these markers.

Thyroid cells express CXCR4

The percentage of CXCR4 expressing cells was determined in bulk cells of all 3 cells lines. There was a significant difference in numbers of CXCR4 positive cells between the N-thy ori-3-1 and the SW1736 cell lines with SW1736 having the highest percentage of positive cells ($p<0.05$) (Figure 6A). However, there was no significant difference in expression of CXCR4 between the N-thy ori-3-1 and the B-CPAP cells (Figure 6A).

Normal Thyroid NSP cells express Thyroglobulin.

Normal thyroid cells secrete the thyroxine hormone, this is modulated by the Thyroglobulin (Tg) gene. sq-PCR was used to determine expression of the Tg gene in sorted SP and NSP cells of the N-thy ori-3-1 and SW1736 cell line. Only NSP cells of both cell lines expressed mRNA for Tg (Figure 6B). GapdH was used as a loading control.

Thyroid SP cells are capable of Colony formation

Sorted SP and NSP cells were assessed for their colony forming unit (CFU) potential. SP and NSP cells from both cells lines were capable of CFU formation but for both cell lines the SP cells had a significantly higher CFU capacity than the NSP. In addition the SP of the SW1736 had a significantly higher CFU formation rate than the N-thy ori-3-1 ($p<0.05$) (Figure 7).

Thyroid SP cells undergo asymmetric division

Asymmetric division potential of sorted SP and NSP from both cells lines was assessed at day 10 post sort. In both cases the SP and NSP were capable of asymmetric division but the SP derived from sorted NSP was lower (Supplementary Figure 3). For the SP sorted from SP, the SP percentage increased, with the SP for N-thy or-3-1 going from 0.25 (±0.15) to 0.45 (±0.25) while for the SW1736 the SP percentage increased from 0.5 (±0.2) to 1.5 (±0.6). Data is presented as mean ±SD, n=2.

ATC SP cells are migratory and invasive and ATC SP migrate in response to CXCL12

We used Boyden chambers migration assays and observed that only a small number of N-thy ori-3-1 SP and NSP were capable of migration and there was no significant difference between the migration abilities of the two populations. However, both NSP and SP of the SW1736 cell line where more migratory than either of the N-thy ori-3-1 cell populations. The SW1736 SP where significantly more migratory than then the SW17236 NSP **(**$p<0.05$) and the SP of the SW1736 had a significantly higher number of migrating cells compared with the N-thy ori-3-1 SP ($p<0.001$) (Figure 8A).

Using a matrigel invasion assay and CXCL12, we observed that only a small percentage of N-thy ori-3-1 SP and none of the N-thy ori-3-1 NSP were capable of invasion, while both cell fractions of the SW1736 where capable of invasion albeit at low levels for the NSP, with the SP invading cells numbers being significantly higher than the NSP ($p<0.001$) and being significantly higher than the invasive N-thy ori-3-1 SP ($p<0.001$) (Figure 8B). We also compared chemotactic invasion to control invasion (control is invasion using complete media not supplemented with CXCL12) of the SW1736 SP cells and noted a significant difference **(**$p<0.05$**)** with SP cells being more invasive in response to chemotactic invasion (Figure 8C).

Discussion.

The SP assay has created a lot of interest as a method of identifying stem cells and CSC in thyroid cell lines and thyroid tissues [5, 10-12]. However, in many studies the SP protocol used has been based on that established by Goodell and colleagues for identification of haematopoietic stem cells from bone marrow [13]. The variability in SP conditions between different cell types and tissues has been demonstrated, and parameters such as Hoechst 33342 dye concentration, incubation time and temperature, the type of ABC-transporter inhibitor and cell viability could all alter the staining pattern and the percentage of reported SP population [14]. In order to improve the accuracy and reproducibility of SP assay, we have optimised the protocol for each thyroid cell line.

We observed that the Hoechst 33342 dye concentration and the length of incubation had a profound effect on SP discrimination. With low concentrations of Hoechst dye (3µg/ml) and shorter exposure time (45 minutes), under-saturated cells were observed that could be mistaken for SP. However, when we used higher concentrations of Hoechst dye (7µg/ml) and a longer incubation time (90 minutes), high cell death rates occurred probably due to dye toxicity, translating to a reduction in SP cell numbers (data not shown). The impact of Hoechst concentration on SP discrimination has been reported previously in a number of studies of both cell line and primary tissue derived SP, however not all have reported on the impact of incubation time. This is, to our knowledge, the first study to report on the impact of both Hoechst concentration and incubation time in thyroid cell lines. The optimal conditions for best SP resolution were found to be different for the three cell lines and represented a balance between the size of SP yield and cell viability for each cell line.

The use of the most appropriate ABC transporter inhibitor allows for the most accurate discrimination of the SP population. ABC-transporters consist of multiple transmembrane helixes and nucleotide-binding domains for ATP hydrolysis that act as xenobiotics. ABCG2, a half-transporter has been associated with multi-drug resistance in breast and other cancers, due to its ability to efflux a number of chemotherapeutic agents, including methotrexate, doxorubicin and paclitaxel [15]. The choice of ABC-transporter inhibitor is guided by the type(s) of ABC transporter thought to confer the SP phenotype on the cells of interest. The ABCG2 transporter was predominantly expressed in N-thy ori-3-1 and BCPAP cell lines with ABCB1 being expressed at a lesser level, whilst the SW1736 lacked expression of ABCB1 but expressed ABCG2. We titrated the dose of verapamil, a calcium-channel blocker [16] which has the affinity to block both transporters, until we observed full inhibition of dye efflux by the cells (100µM) without causing high levels of cell death. Doses above this resulted in higher levels of cell death. Interestingly, when all cell lines where treated with FTC, a specific inhibitor of ABCG2 [17, 18] a small fraction of SP persisted in all cases. This may suggest that FTC concentration was not adequate in achieving full saturation of the ABC transporters expressed by these cells. However, this may also be indicative of the possible involvement of other transporter subtypes in these cell lines such as ABCC1, ABCC5, ABCB11 and ABCA2 that are also capable of mediating the SP phenotype [19, 20].

The SP assay is a dynamic assay, which requires intracellular processes to be intact for the cells to be able to expel Hoechst dye. Reagents used in the assay include cell dissociation agents, inhibitors and Hoechst 33342 dye all of which are toxic to the cells and steps to minimise cell death should be taken at every stage of the SP procedure. For example we observed that when using trypsin-EDTA to detach BCPAP cells from tissue culture plastic, cell viability (measured as part of the SP assay, data not shown) was low (49.5 percent), but we were able to reduce this cell loss by using Accumax (65.7 percent) to detach the cells. This may be in part due to the nature of the reagents used, Accumax is a commercially available cell detachment reagent that consists of proteolytic and collagenolytic enzymes while trypsin is a member of the serine protease family and cleaves peptides on the c-terminal side of Lysine or

arginine and EDTA is a divalent cations chelator; both are used to weaken cell attachment to tissue culture plastics and have variable effects on different cell types.

We observed that with both the normal and thyroid cancer cell lines, cell confluency affected the SP percentage, lower confluency having higher SP. It has previously been reported that cell confluency has an impact on SP cell numbers of cells of the SW480 and DLD1 colon cancer cell lines and MCF7 breast cancer cell line, with cells plated at lower densities having a higher SP when harvested. [21]. This increase in SP cell numbers with lower confluency has been suggested to be due to two potential mechanisms, stress resulting in a proliferate response or a lack of cell to cell contact triggering the self-renewal pathway [21].

In addition we examined bulk cells of the N-thy ori-3-1, BCPAP and SW1736 cell lines for expression of the C-X-C chemokine receptor type 4 (CXCR4), which we have previously reported to be important for SP cell migration in normal tissues [22-23]. There was significantly higher expression of CXCR4 in the SW1736 cell line compare to the N-thy ori-3-1 cell line but there was no significant difference between the latter and the BCPAP in terms of CXCR4 expression. Downregulation of CXCR4 has been shown to reduce the migratory ability of colorectal cancer cells and breast cancer cells [24, 25], therefore we hypothesized that as there was no significant different in CXCR4 expression between the normal and BCPAP cells this would support less difference in the migratory abilities of both cell populations. Therefore, for this study, we focused on the differences between the normal thyroid and ATC thyroid cell line, as we had already observed a significant difference in CXCR4 expression and migratory potential.

We also examined the N-thy ori-3-1 and SW1736 SP and NSP cells, showing that while both SP and NSP were capable of CFU formation, asymmetric division and expressed stem cell markers, in both cases the SP cells displayed higher levels of these stem cell characteristics than the NSP cells. In addition there was a trend for the SW1736 SP to express higher levels of stem cell markers that the N-thy ori-3-1 SP, although OCT4 was the only marker that was significantly more highly expressed. Why there was no significant difference in expression of SOX2 protein between the SP of both cell lines may be due to the fact that, while in many cancers SOX2 mRNA and protein levels have been reported to be increased in poorly differentiated types [26], this is not the case for all cancers. In human gastric carcinoma SOX2 protein expression was reported to be higher in the normal gastric mucosa and intestinal metaplasia than in the gastric carcinoma [27]. While in another study of human gastric cancer SOX2 protein was reported to be reduced in most gastric cancers compared with that of corresponding normal tissue [28]. These studies suggest that while SOX2 is important in both stem cells and CSC, its level of expression needs to be further understood, and may be variable amongst different types of cancers in different organs. Therefore, while the SP of both the SW1736 and N-thy-Ori-3-1 both showed significant differences in protein expression of SOX2 between themselves and their NSP, the fact that they do not show a significant difference in levels of SOX2 protein expression between normal versus ATC SP may be related to the balanced relationship between SOX2 and its many downstream targets, something that has not as yet been well explored within the context of ATC. However, we did see a significant difference in gene expression between the bulk normal and SW1736 cells with OCT4 being significantly more highly expressed in the SW1736 cells (reflecting the protein expression seen in our ICC study). Moreover, we also detected significantly higher expression of SOX2 mRNA in the bulk SW1736 compared to the N-thy-Ori-3-1, but in this study this did not correlate to a significant difference in SOX2 protein expression between the SP of the two cell lines. However, it is now recognised that reliance on mRNA levels to predict protein abundance is not sufficient and this might in part account for the differences we observed between protein and gene expression [29].

In a study of the HeyA8MDR paclitaxel-resistant ovarian cancer cell line we observed that NSP can sometimes give rise to SP and NSP and hypothesized this may have been due to the presence of other stem cell populations within this cell line [2]. This may also be the case for the cell lines used in the present study. Numerous markers have been reported to identify putative thyroid CSC e.g. CD44+CD24-, CD133, CD15 [30]. Normal thyroid stem cells have also been reported to be identifiable based on expression of a range of markers e.g. p63, CD34+/CD45- [31]. How much overlap there is between these various cancer and normal stem cell populations with SP, if any, remains unexplored, but more than one stem cell population can exist within a single tissue and this might account for the ability of NSP cells to give rise to SP.

In addition, we examined the SP and NSP cells of both cells line for expression of Tg which plays a crucial role in production of thyroxine, secreted by the follicular cells. We observed that for both cells lines only the NSP cells expressed Tg, while the SP cells showed no expression, this is probably due to the SP cells being undifferentiated stem cells. It has previously been reported in studies of embryonic and induced pluripotent stem cells that expression of Tg is linked with stem cell differentiation [32, 33]. Tg mRNA expression has previously been reported to be expressed by normal thyroid and to be absent in bulk SW1736 cells [29]. However, in another study using the ATC cell line THJ-11T, low levels of Tg protein were detected [34]. Why Tg is sometimes detected in ATC cell lines and not in others remains unclear, this may be due to differences in techniques used, but it is worth noting that Tg expressing cells can be found in small numbers in some ATC [35].
We also examined the ability of both the SP and NSP cells of both cell lines to migrate and while all 4 cell populations demonstrated some migration potential it was lower in both NSP and there was no significant difference in migration between the N-thy ori-3-1 SP and NSP, the SW1736 SP cells had the highest migration potential. Analysis of the chemotactic invasive capacities of both cell lines SP and NSP cells clearly demonstrated that the SW1736 SP where highly invasive compared with their NSP and with the N-thy ori-3-1 SP. We further confirmed the importance of the CXCR4/CXCL12 axis in invasion of the SW1736 SP, showing significant invasive cell numbers towards CXCL12 than under control conditions. The importance of the CXCR4/CXCL12 axis in cancer progression has been clearly established for a number of cancers, while its role in thyroid cancer is still being explored. An *in vivo* study has shown that use of the CXCR4 specific antagonist AMD3100 only reduced rather than abrogated tumour growth in a mouse model of undifferentiated thyroid cancer [36], suggesting that targeting the CXCR4/CXCL12 axis might only slow undifferentiated thyroid progression. A recent study has suggested that in thyroid cancer the CXCR4/CXCL12/CXCR7 axis may be important for thyroid cancer development, this requires further exploration but suggests that this axis causes thyroid cancer cells to become more migratory and invasive [37].
We have previously reported on the presence of SP cells in the N-thy ori-3-1, BCPAP and SW1736 cell lines [38]. However, we did not reported on the optimisation of the SP protocol in this previous study, instead we focused on the impact of hypoxia on the SP. Hypoxia exposure resulted in a decrease in SP cells in the normal thyroid cell line and an increase in SP of both cancer cell lines. Again this differential response of normal SP and cancer SP to hypoxia, has the potential to lead to new treatment targets for thyroid cancer e.g. the hypoxia inducible pathway.
In conclusion, the results of our study suggest that while normal thyroid SP and thyroid cancer SP cells are similar, ATC SP cells are more migratory and invasive and potentially contribute to ATC progression. In addition, our findings support a role for CXCR4 and CXCL12 in the ATC SP cells ability to migrate and invade. However, further studies are required to delineating differences in the regulatory pathways between SP cells of normal thyroid and ATC to enable identification of potential therapeutic targets of ATC SP that do not target normal thyroid SP.

Experimental procedures

Cell lines

Normal thyroid cell line, N-thy ori-3-1 was purchased from the European Collection of Cell Cultures [ECACC, London, UK], while the papillary, BCPAP and anaplastic, SW1736 cell lines were kind gifts from Professor G. Brabant (Universitats Klinikum, Lubeck, Germany) and Professor McCabe (University of Birmingham, UK) respectively. Authenticity of all cell lines used was confirmed on the 21st of December 2015 by Short tandem repeat (STR) fingerprinting performed by ECACC.

Culturing and propagation of cell lines.

The N-thy ori-3-1 and BCPAP cell lines were cultured in the RPMI media [Gibco], whilst the SW1736 cells were cultured in DMEM+GlutaMAX media [Gibco]. All media contained 10% FBS, 2mM L-glutamine [Sigma] and penicillin/streptomycin 5000 units [Gibco]. Media was changed every 3 days. Cells were detached and passaged at 80% confluency using 0.05% trypsin-EDTA [Gibco] for a maximum duration of 3 minutes with the exception of the BCPAP cell line that was detached using Accumax for 3 minutes [Innovative Cell Technologies, California USA]. Abrupt inactivation of trypsin

or Accumax was performed by adding cell suspension to media containing 10% FBS. The cell suspension was then pelleted by centrifuged for 5 minutes at 3000rpm, resuspended and counted on a Neubauer haemocytometer [Neubauer, Scientific Laboratory Supplies, Nottingham UK]. Cells were then either re-plated for further expansion or used directly for analysis.

Semi-Quantitative PCR for ABC transporter and Thyroglobulin (Tg) expression

RNA from the cell lines was extracted using RNeasy micro plus kit [Qiagen, Manchester UK], as per manufacturer's instructions. RNA concentration was measured using a Nanodrop UV-vis Spectrophotometer 2000 [Thermo Scientific, Paisley UK], and RNA integrity was checked using 2% Agarose gel electrophoresis. cDNA synthesis was performed using a Tetro-cDNA synthesis kit [Bioline, London, UK] as per manufacturer's instructions. Semi-quantitative PCR was performed as described previously [2]. Primers used included ABCG2; F:5'-GAGCGCACGCATCCTGAGAT-3',R:5'- TCATTGGAAGCTGTCGCGGG-3';ABCB1;F:5'-CTGACGTCATCGCTGGTTTC-3',R:5'-ATTTCCTGCTGTCTGCATTGTGA-3'; Tg; F:5'-GCTGTCCGAGACCTCTGTTT-3',R:5'-AGAGATTCCTGGCTTCCGGT-3'; GapdH; F:5'- GCCTTCTCCATGGTGGTGGTGAA-3', R:5'-GCACCGTCAAGGCTGAGAAC-3', was used as a loading control.

Quantitative PCR to determine expression of stem cell markers in normal and thyroid cancer cells

Quantitative PCR (qPCR) was carried out as described previously [2]. The reaction was set up at 95^0C for 10 minutes followed by 40 cycles of 95^0C for 15 seconds and 60^0C for 1 minute. GapdH was used as an internal control and for normalisation. Taqman primer probes where purchased from Thermofisher [Thermofisher, UK].

To determine optimal ABC transporter inhibitors to prevent Hoechst dye efflux by normal thyroid and thyroid cancer cells

Cells were resuspended in either RPMI [Gibco] at $1x10^6$/ml for N thy ori-3-1 and BCPAP; or in DMEM+GlutaMAX [Gibco] for SW1736, all media contained 2% FBS [Gibco]. Based on the standard PCR results for ABC mRNA expression (Figure 3A), either Verapamil [Sigma-Aldrich, Gillingham, UK] or Fumitremorgin C (FTC) [Axxora, Farmingdale, NY, USA] was added to the control samples (Figure 1). Varying concentrations of Verapamil (50μM, 100μM, 150μM, 200μM and 250μM) or FTC (30μM, 50μM and 70μM) were used and samples incubated rotating on a MACsMix rotator [Miltenyi Biotec, Bisley, Surrey UK] in the dark at 37^0C for 15 minutes.

To determine optimal Hoechst 33342 dye concentration and incubation time

Following inhibition of ABC transporters, all samples were then incubated with different concentrations (3, 5 or 7μg/mL) of Hoechst 33342 [Sigma-Aldrich) for 45, 60 or 90 min. All reactions were terminated by adding 30mL of cold PBS and washed once. Cell pellets were resuspended in cold PBS (500μL) before being filtered through a 70 micron cell strainer [Biologix Group Ltd, Shandong, China] into 5ml polypropylene tubes [BD Falcon].

Flow cytometry analysis

After Hoechst staining and immediately prior to flow cytometric analysis, 2μg/mL propidium iodide (PI) [Sigma-Aldrich] was added to all cell samples. Cells were analysed using a LSRII flow cytometer (BD). Gating strategy used was as described previously [9]. Data was analysed using BD FACSDiva software version 8 [BD].

Cell confluency

For all cell lines, cells were counted and plated at densities of 0.25, 0.5, 1.0 and 1.5 X 10^6/ml, and cultured as described above, for 3 days, SP analysis was then performed using the optimised SP protocol. For analysis, SP from a total of three replicates were determined and mean SP was plotted against plating densities for each cell line.

Colony forming potential

5,000 sorted SP and NSP were plated into a 6-well plate [Corning Inc., Kennebunk USA] and allowed to grow. Two mL of media was added every 4 days. Images of colonies formed were taken using an

inverted phase contrast Axiovert 200M microscope [Zeiss] at day 10 after plating. Number of colonies was counted from five independent areas within each well.

Asymmetric division

10,000 sorted SP and NSP cells were grown for 14 days before each was subjected to SP analysis using our optimised protocol. The percentage of SP to NSP between the normal thyroid and anaplastic cancer cells were analysed and compared. Two biological replicates of SP and NSP cells were analysed in total.

Immunocytochemical analysis for expression of Nanog, Oct4 and Sox2

200 sorted SP and NSP cells were collected directly onto poly-L-lysine coated slides [Fisher Scientific, UK] and fixed in ice cold methanol [BDH Lab Supplies] at -20 ^{0}C for 20 minutes. Slides were then washed with 1xPBS for 5 minutes and cell membranes were permeabilised with 0.3% (v/v) Triton X-100 [Fisher Scientific, UK] diluted in PBS for 10 minutes. Blockage of the non-specific binding sites was achieved using 5% normal goat serum (NGS) made up in PBS for 30 minutes in a humid chamber. Next, slides were incubated in primary antibodies diluted in 0.05% NGS/PBS for one hour in a humid chamber in the dark.

Slides were then washed with 1xPBS, 3 times for 5 minutes, before being incubated in the appropriate secondary antibody (secondary antibodies used in this study where FITC-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody [Jackson Immuno Research Lab, Inc] diluted 1:25 in 0.05% NGS/PBS for 30 minutes in the dark. Finally, slides were mounted in vectashield with 4', 6-diamino-2-phenylindole (DAPI) [Vector Lab] and covered with a cover slip. The negative control slides were treated as above but stained only with the secondary antibody. Primary antibodies used in this study were anti-Sox2 rabbit polyclonal, dilution 1:100 [Abcam], anti-Oct4 mouse monoclonal, dilution 1:100 [BD bioscience] and anti-Nanog mouse monoclonal, dilution 1:100 [Abcam].

Mean fluorescence intensity

Images were taken at X20 magnification and each cell population was imaged at five different arbitrary regions. Analysis was performed using Image J version 1.44. Fluorescence intensity was measured by subtracting the fluorescence intensity of each defined cell minus the background intensity measured immediately outside the cell of interest. Mean value for all measured cells (±SD) was taken as the arbitrary unit for mean fluorescence intensity (MFI) and compared between SP and NSP. Information on statistical analysis used is shown below.

CXCR4 expression

Three biological replicates of 200,000 bulk cells from each cell lines were stained with APC conjugated CXCR4 antibody [R&D Systems] for one hour. Cells were washed twice before being suspended in FACS buffer and analysed using BD FACS Canto Analyser and FACS
Diva software [BD Biosciences].

Crystal violet motility and invasion assay

Motility and invasion assays were performed using 8 µM Transwell chambers [Corning] with and without Matrigel coating respectively. For the migration assay, 10,000 cells were plated into the upper chamber in serum free media and complete media was used to fill up the lower chamber. For the invasion assay, 5000 cells were plated into the upper chamber in a similar manner to that for migration, whilst the lower chamber was filled with either complete media or complete media with the addition of 100ng/ml CXCL12 [R&D Systems]. After 22 hours, non-migrated cells were removed using cotton swabs and the cells attached to the lower part of the chamber were fixed using 75% cold ethanol. Fixed cells were then stained with 0.1% (w/v) crystal violet [Sigma] for 10 minutes. Cells were counted from images taken from five independent chamber areas using an inverted phase contrast Axiovert 200M microscope [Zeiss] and processed using AxioVision40 version 4.8.2.0 software [Zeiss]. Three biological replicates from SP and NSP of normal and anaplastic thyroid cells were studied. Cells incubated in chambers without the addition of CXCL12 were used to represent baseline invasion.

Statistical analysis.

Results were expressed by means ± SD and statistical analysis was performed using IBM SPSS Statistics for Windows, version 23 [IBM Corp., Armonk, N.Y., USA]. Parametric t-test and one way ANOVA were used for normally distributed data whilst Mann-Whitney U and Kruskal-Wallis tests were used for skewed data. Results with p-value of less than 0.05 were considered statistically significant. In all statistical analysis: *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

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Conflict of interest statement*:* The authors have nothing to disclose

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Figures

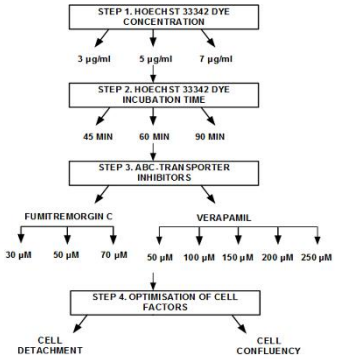


Figure 1. Flowchart showing step taken to determine the optimal procedures for the side-population assay for use with normal and cancer thyroid cell lines.

A

	N-THY ORI 3-1	BCPAP	SW 1736
OPTIMAL HOECHST CONCENTRATION (UG/ML)	7	5	5
OPTIMAL INCUBATION TIME (MIN)	90	60	90

B

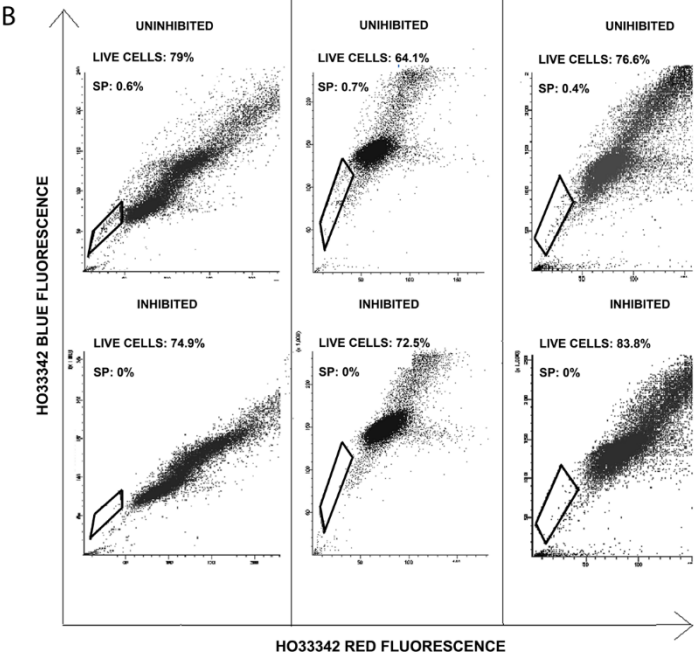


Figure 2. Optimal Hoechst 33342 dye concentration and incubation time for cells in contact with dye. Optimal dye concentration and incubation are given in (2A). Representative FACS profiles of SP in N-

thy ori-3-1, BCPAP and SW1736 using these optimal conditions are shown in (2B). Note the cell viability is given for each sample and note the presence of SP cells in the gated region and their absence on the addition of verapamil. Also note addition of the inhibitor does not negatively affect cell viability.

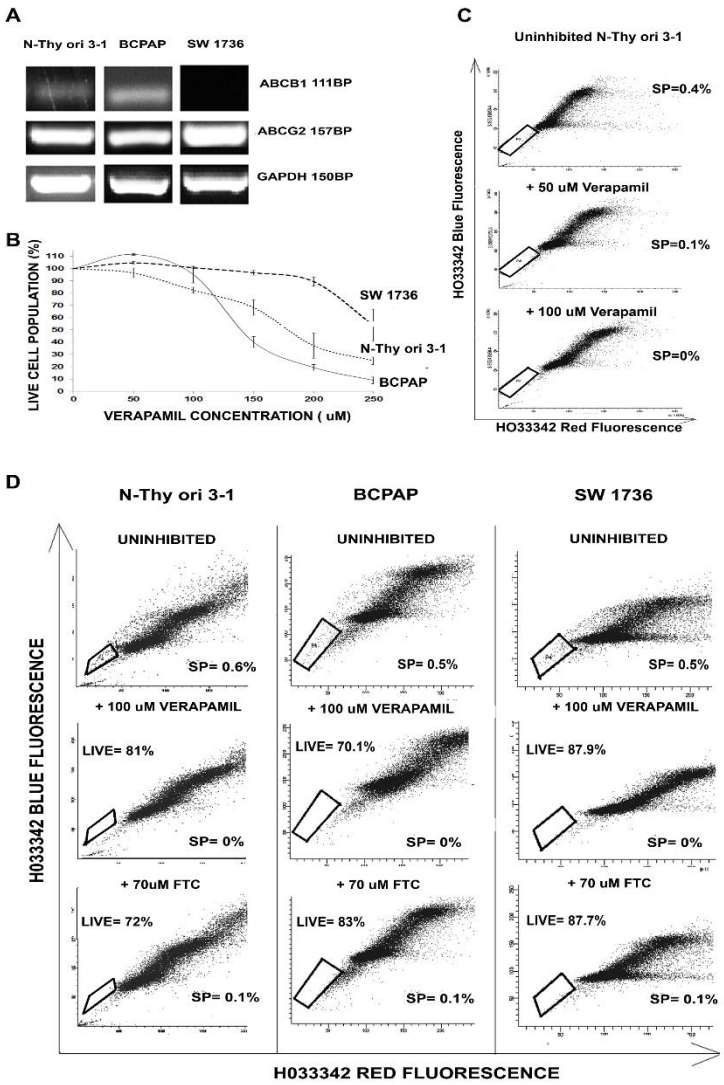


Figure 3. Optimisation of ABC-transporter inhibitors for thyroid SP assay.
To determine which transporter might be expressed we examined all 3 cells lines for ABCB1 and ABCG2 expression. Representative images of PCR results showing mRNA expression of ABC transporter in thyroid cell lines (A). Note N-thy ori-3-1 and BCPAP express both transports whilst SW1736 only expresses ABCG2. GapdH was used as a loading control. Assessment of Verapamil-induced cell toxicity in bulk cells of each cell lines is shown in (B), note decreasing live cell percentage with increasing concentrations of verapamil. Representative FACS plots showing SP discrimination with different concentrations of verapamil in the N-thy ori-3-1 cell line SP (C) note the residual SP population when using 50 µM of verapamil versus the absence of cells capable of efflux in the SP gate when using 100uM of verapamil. Representative FACS plots showing SP discrimination using different agents to inhibit ABC-transporters (D), note that all SP of all 3 lines show complete inhibition of dye efflux with 100µM of verapamil but all show residual SP in the presence of FTC at 70uM.

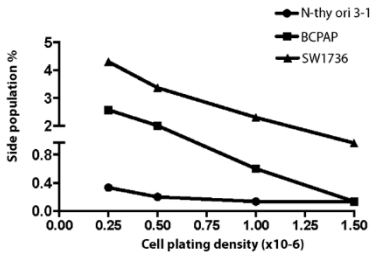


Figure 4. Graphical representation showing SP cell percentage in relation to cell confluency. Reduction in percentages of SP is seen in all three types of thyroid cell lines when plated at increasing cell number and analysed after three days using the optimised SP assay. Note that the normal thyroid cell line has the lowest subpopulation of SP. In N-thy ori-3-1 cell line the SP drops from 0.3 to 0.1%, B-CPAP from 2.6-0.1% and in SW1736 cells from 4.3% to 0.9%. N=3 for all cell lines and means are shown as representatives.

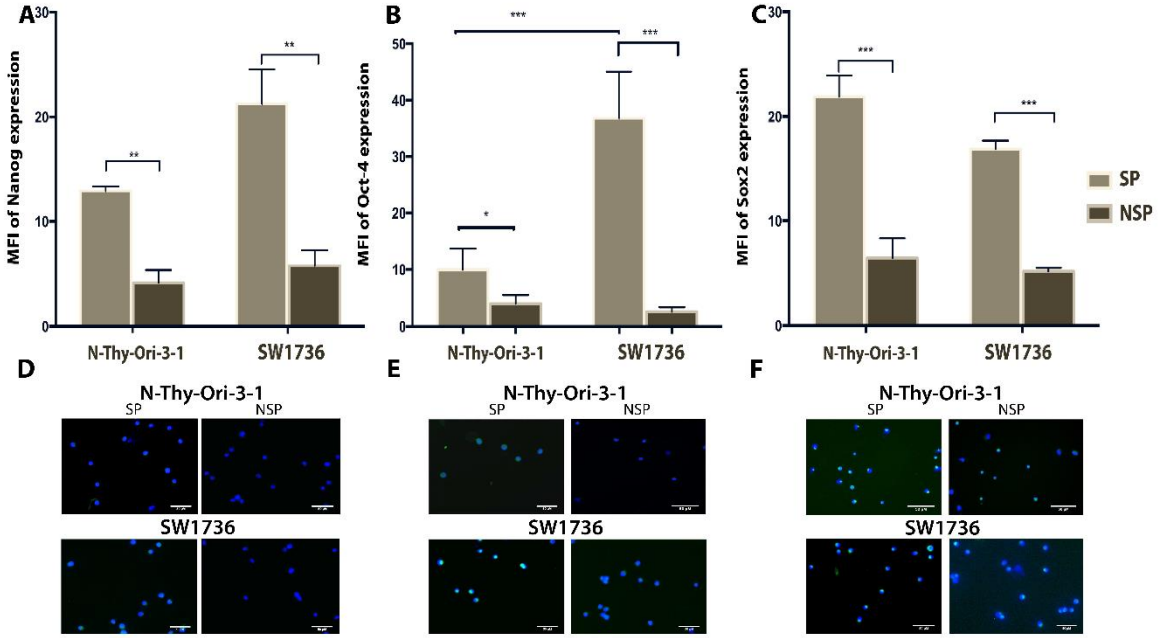


Figure 5. Normal thyroid and anaplastic thyroid cancer SP cells express stem cell markers. ICC analysis was used to identify the number of cells expressing NANOG, OCT4 and SOX2 and the MFI determined for NSP and SP cells. Both SP cells had significantly higher expression of NANOG (5A) ($p<0.01$) and (5B) OCT4 (for N-thy ori-3-1 $p<0.05$ and for SW1736 $p<0.001$) than the NSP cells. In addition SW1736 SP cells had significantly higher expression of OCT4 compared to the SP of normal thyroid cells (MFI means 36.7691 ± 2.1316 vs 9.9770 ± 1.2235) ($p<0.001$) (5B). Both SP cell populations had significantly higher expression of SOX2 than their counterpart NSP cells (5C) (N-thy ori-3-1 $p<0.001$ and SW1736 $p<0.001$), (MFI mean 21.884 ± 1.5911vs 6.4702 ± 4.6720 for N-thy ori-3-1 cells and 16.8527 ± 6.9479 vs 5.1483 ± 4.0068 for SW1736 cells), MFI, Mean fluorescent index. N=3. Representative immunofluorescence images are shown for sorted cells from both cells lines stained for expression of Nanog (D), OCT4 (E) and SOX2 (F) Antibodies-FITC=green, cell nuclei DAPI=blue.

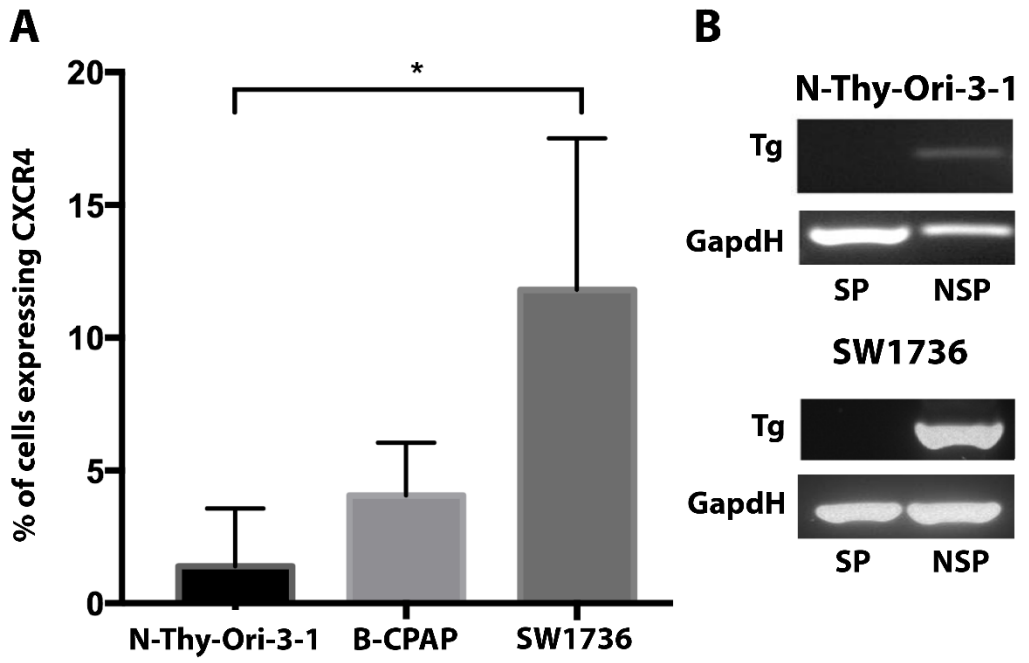


Figure 6: Protein expression of CXCR4 and mRNA expression of Thyroglobulin. (A) Bar chart representing flow cytometric analysis results for percentage of cells expressing CXCR4 in unsorted N-thy ori-3-1, B-CPAP and SW1736 thyroid cell lines. SW1736 cells had the highest number of CXCR4 expressing cells (mean % of positive cells = 11.8% ± 4.656) and this was significant ($p<0.05$) when compared to the N-thy ori-3-1 normal thyroid cells (mean % of positive cells = 1.4 ±1.77). The B-CPAP contain fewer CXCR4 expressing cells than the SW1736 but more CXCR4 expressing cells than the N-thy ori-3-1 (B-CPAP mean % of positive cells = 4.2% ± 1.18) but this was not significant. N=3. Representative images for results for mRNA expression of Thyroglobulin (N=3) (6B). Note that Thyroglobulin expression can be detected in the NSP but not in the SP of both cell lines. GadpH was used as a loading control.

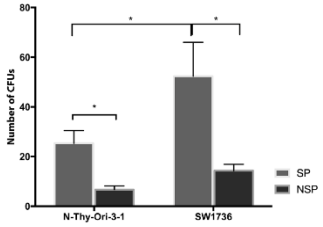


Figure 7: Normal thyroid and anaplastic thyroid cancer SP cells can form colonies. Bar chart representing quantification of colony forming units (CFU) at day 10 in culture. CFU formation was significantly higher for SP compared with NSP in both cell lines ($p<0.05$) and, between SP cells, for SW1736 compared with N-thy ori-3-1 ($p<0.05$). CFU, colony forming unit. N=3.

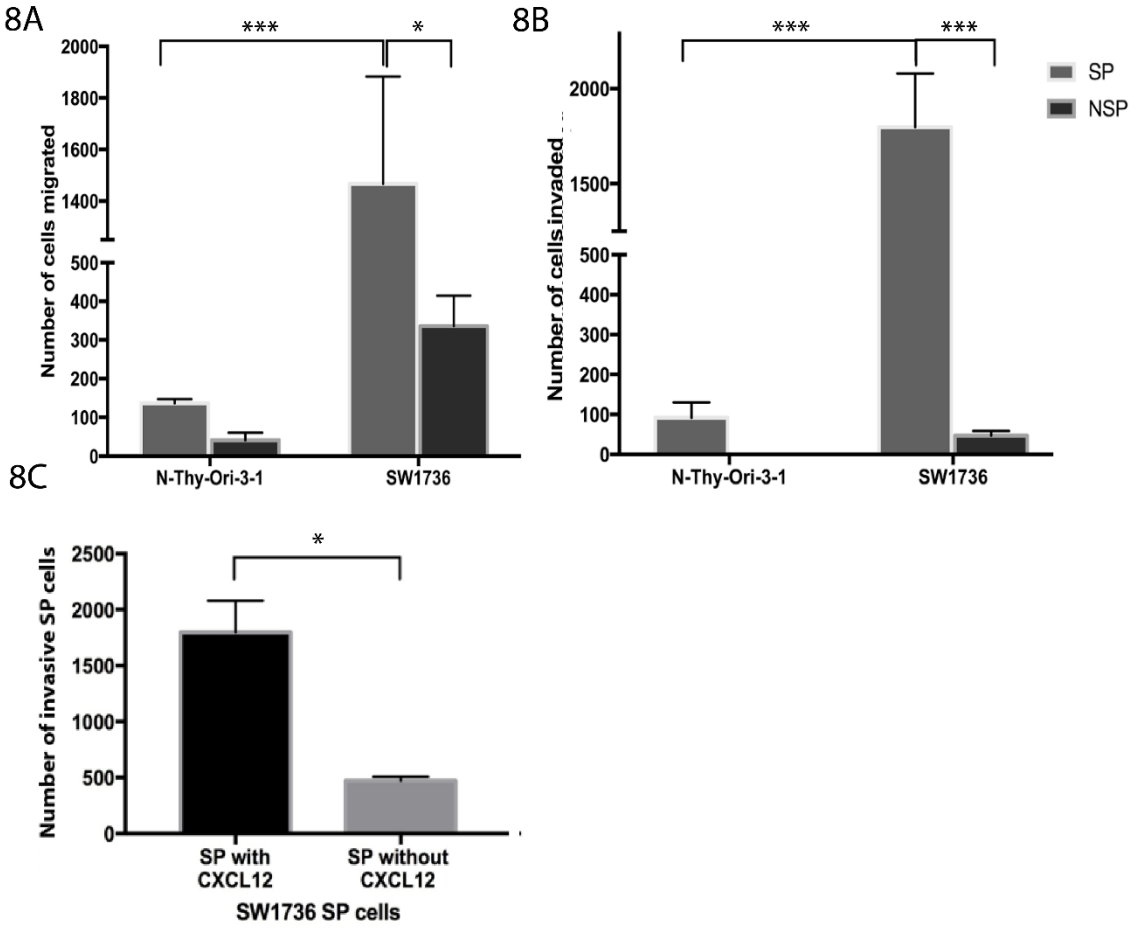
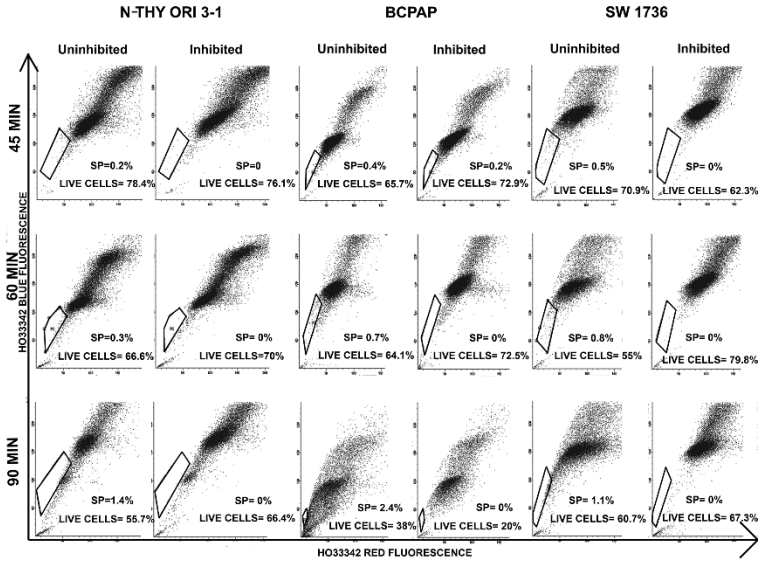
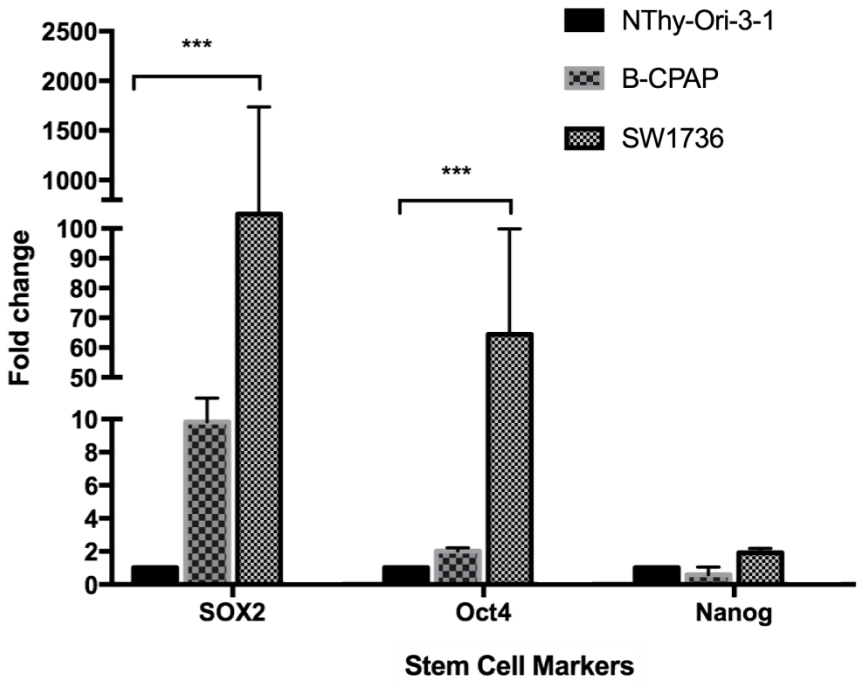


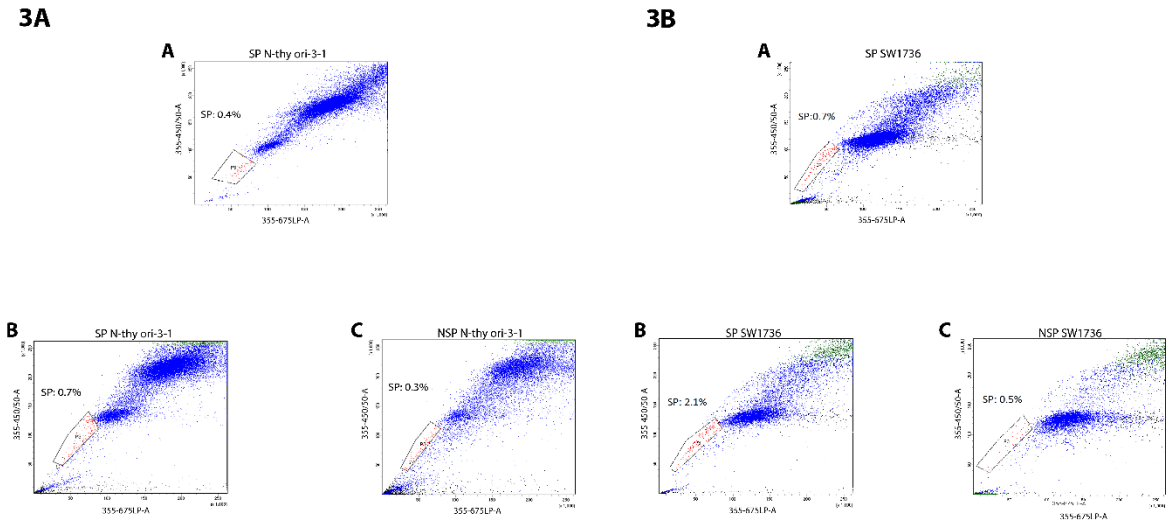
Figure 8: Anaplastic thyroid cancer SP cells are more migratory and invasive than normal thyroid SP. Bar chart representing analysis of number of migrated SP and NSP cells of N-thy ori-3-1 and SW1736 (8A). SP and NSP were incubated in Boyden chambers for 22 hours and then the number of migrated cells where counted. Note the significant difference in migrated normal thyroid (mean ± SD 136.67 ± 8.654) and anaplastic thyroid cancer (mean ± SD 1466.67 ± 339.934) SP cells ($p<0.001$). N=3. Bar chart representing number of SP and NSP cells of N-thy ori-3-1 and SW1736 that invaded through matrigel (8B). SP and NSP were incubated in Boyden chambers coated with matrigel for 22 hours and then the invasive cells where counted. Note in both cases the SP were more invasive than the NSP and none of the normal thyroid NSP cells were capable of invasion. Also note that the SW1736 SP cells were significantly more invasive than the N-thy ori-3-1-SP cells (mean of number of cells that had invaded ± SD 1796.667 ± 230.988 vs 92 ± 31.156) ($p<0.001$). N=3. (8C) CXCL12 enhances invasion of anaplastic thyroid cancer SP cells. SW1736 SP that invaded through the Matrigel coated membrane were counted for both control (media not supplemented with CXCL12) and CXCL12 treated SP. A significantly higher number of SP cells invaded through the Matrigel-coated membrane when CXCL12 was added to the invasion assay compared to control ($p<0.05$). N=3.



Supplementary figure 1. Representative FACS plots for N-thy ori 3-1, BCPAP and SW1736 incubated with optimum concentration of Hoechst 33342 dye (N-thy ori 3-1, 7µg/ml, BCPAP 5µg/ml and SW1736 5µg/ml) for 45, 60 and 90 mins. Different incubation times have an effect of both SP profile and SP percentage.



Supplementary Figure 2. Graphical representation of qPCR data showing expression of mRNA levels of stem cell markers in bulk cells of the N-thy ori 3-1, BCPAP and SW1736 cell lines. Levels were measured as fold change to the normal thyroid gene expression and normalised to GapdH. Note the significant difference in SOX2 and OCT4 expression between the SW1736 and N-thy ori 3-1 cells.



Supplementary Figure 3. Representative images of FACS analysis showing asymmetric division of N-thy ori-3-1 (2A) and SW1736 anaplastic thyroid cancer cells (2B). For both cells lines SP and NSP cells were sorted from Hoechst 33342 stained cells primary culture shown in (A). Note profiles after culture for 10 days of sorted SP (B) and sorted NSP (C), for both cells lines SP gave rise to both NSP and SP (B), but the SP populations were bigger than those of the starting cultures and in both cases the NSP could also give rise to SP and NSP (C) but the SP numbers were smaller than those derived from the SP only cultures. N=2.